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# Effects of Chrysotile Co-exposure on BaP Binding in Normal Human Fibroblasts

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The effects of chrysotile and benzo(a)pyrene (BaP) co-exposure on uptake, metabolism and BaP binding to nucleic acids were investigated in normal human fibroblasts. A significant enhancement in these parameters was found when co-exposure was carried out under serum-free conditions. In the presence of serum, only a slight enhancement of BaP uptake was detected, while metabolism and binding to nucleic acids were not affected over the 8-hr incubation period studied.

## Introduction

In 1977, Saracci noted that asbestos and cigarette smoking may act synergistically or even multiplicatively to produce lung cancer (*1*). Results reported by Hammond et al. (*2*) on relative risk analyses of a large group of North American insulation workers supported the multiplicative model. Later, data collected on chrysotile miners and millers from Quebec and reported by McDonald et al. (*3*) supported a more than additive but less than multiplicative synergistic effect of asbestos and cigarette smoking.

Benzo(a)pyrene (BaP) is one of several carcinogenic components in cigarette smoke that have been implicated in human respiratory neoplasms (*4*). The carcinogenic action of BaP in experimental animals has been hypothesized to act through its ability to bind to cellular DNA (*5*). On the other hand, the mechanism by which asbestos exposure initiates pulmonary tumors is unknown. It has been suggested that asbestos may facilitate the phenotypic expression of a transformed cell through the stimulation of cellular proliferation (*6*).

An enhanced uptake of BaP into biological systems in the presence of asbestos has recently been reported by this and other laboratories (*7 – 9*). This study examined the effect of chrysotile co-exposure on the uptake, metabolism and binding of BaP in normal human fibroblast cell cultures in both the presence and absence of serum and concluded that in the presence of asbestos, a higher level of BaP is taken up by the cell and is thus available for metabolic activation and subsequent macromolecular binding.

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## Materials and Methods

### Cell Cultures

Normal human skin fibroblasts were cultivated as described previously (*6*). Karyotype was checked and shown to have a normal human chromosomal pattern. Mycoplasm contamination was routinely screened for by the Microbiology Services Division of the National Center for Toxicological Research and consistently found to be negative. At 24 hr following the seeding of $4.2 \times 10^6$ fibroblasts per 100 mm diameter Petri dish, or 24 hr after changing the medium of a confluent fibroblast culture of the same density, growth medium was removed and replaced with one containing 3 μg/mL $^3$H-BaP (New England Nuclear, Boston, MA, specific activity = 50 Ci/mmole was diluted with Florisil purified nonradioactive BaP to yield a specific activity of 211 mCi/mmole) and 10 μg/mL chrysotile (NIEHS, mean width: 0.17 μm, mean length: 21 μm) with or without 10% fetal bovine serum (FBS, Gibco Laboratories, Grand Island, NY) for 8 hr. Three plates per sample group per experiment were used. A viable cell count was performed on each sample by the trypan blue exclusion method as previously reported (*7*).

### Organic Solvent Extraction of Growth Medium

The culture growth medium containing $^3$H-BaP and its metabolites was collected after 8 hr of incuba-

tion. An aliquot of medium of each sample was vigorously mixed with an equal volume of acetone in order to denature any residual protein. Subsequently, an equal volume of ethyl acetate was added to the extraction mixture. The extraction was completed with further vigorous mixing and by centrifugation of 3/4 of the full speed of an IEC table top centrifuge, Model HN-S (approximately 2000 rpm). The organic and aqueous phases were collected separately. The radioactivity of each phase was determined by counting an aliquot of it in the scintillant Aquassure (New England Nuclear) with a Packard 3380 liquid scintillation spectrometer.

### Macromolecular Binding of $^3$H-BaP

Cell cultures at the end of the incubation period were washed three times with phosphate-buffered saline (PBS). Subsequently, cells were trypsinized with 2 mL of 0.01% trypsin in 0.02% ethylenediaminetetraacetic acid (EDTA, Fisher Scientific, Fair Lawn, NJ) for 3-5 min (*7*). Trypsinization was terminated by adding 3 mL of a growth medium containing 10% FBS. A well-dispersed 0.1 mL cell suspension per plate out of a total volume of 5 mL was used for a viable cell count. The rest of the cells were collected by low speed centrifugation (1000 *g*). The cell pellet was washed once with SSC (0.15 *M* NaCl and 0.015 *M* sodium citrate) at 4°C and then resuspended in 0.1 × SSC for 30 min to release the unbound $^3$H-BaP before another low speed centrifugation. The precipitate was then dissolved in 1% sodium dodecyl sulfate (SDS, Sigma, St. Louis, MO) in SSC. Nucleic acids (RNA and DNA) were extracted from the SDS-dissolved solution by first making it 2 *M* in NaCl followed by an extraction with an equal volume of $CHCl_3$:isoamyl alcohol (24:1) (*10*). The aqueous phase, which contained nucleic acids, was collected after a centrifugation at 2000 *g* for 12 min. RNA was hydrolyzed with 0.3 *N* KOH at 37°C for 18 hr. At the end of the hydrolysis, the solution was acidified in an ice-water bath with cold 50% perchloric acid (PCA, Mallinckrodt, St. Louis, MO) to a pH between 2 and 5 (*11*). The precipitate was further extracted for DNA with a 5% PCA solution at 80°C for 45 min. The nucleic acids were quantitated by ultraviolet absorption at 260 nm. The radioactivities of the nucleic acids were determined by liquid scintillation spectrometry as described above.

## Results and Discussion

Cotreatment with chrysotile (10 μg/mL) and BaP (3 μg/mL) led to no observable cytotoxicity after 8 hr of incubation in reseeded high density cell cultures but did slightly induce cell killing in the confluent cell cultures under serum-free conditions (Table 1). The cell densities in these two cultures were approximately equal at the beginning of the experiment (50,000 cells/cm$^2$ dish), but the percent of mitotic figures was less under continuous confluent culture conditions. By analyzing the total radioactivity left in the medium at the end of 8 hr of incubation, we found that in the presence of 10% FBS, the uptake of $^3$H-BaP was essentially the same in control and chrysotile-treated reseeded cultures. An enhanced uptake of labeled BaP in the continuous confluent chrysotile-treated cultures was also observed (Table 1, $p < 0.02$). Approximately twice as much of the $^3$H-BaP was taken up by the control cultures in the absence of serum. However, the presence of 10 μg/mL chrysotile further enhanced uptake by approximately 35-40% over control levels. A similar observation was reported earlier (*7*), when a lower cell density (0.1 × 10$^6$/dish) and a longer incubation time (24 hr) were used. As would be expected due to the low capacity of normal human skin fibroblasts to metabolize BaP (*12*), when an aliquot of the collected growth medium was extracted with organic solvents, the major part of the radioactivity measured in the organic phase was due to the nonmetabolized $^3$H-BaP. Indeed, as shown in Table 2, 70-80% of the radioactivity from the serum-free medium was extracted into the organic phase. While the recovery of radioactivity in the serum-containing medium was incomplete, only 7-8% of the total applied radioactivity was converted to water soluble-metabolites including sulfate esters, glucuronides and glutathione conjugates (*13*) and the other organic solvent-extractable metabolites, including minute quantities of diols, quinones and phenols (*13*). The less than 100% recovery of radioactivity in the organic and aqueous phases of the

Table 1. Effects of chrysotile cotreatment on $^3$H-BaP uptake by and viability of normal human fibroblasts.[a]

| Culture | Serum | Radioactivity left in medium, % | | Viable cells × $10^{-5}$ | |
|---|---|---|---|---|---|
| | | Control | Chrysotile | Control | Chrysotile |
| Reseeded | + | 76.3 ± 1.4 | 76.3 ± 1.6 | 30.6 ± 2.0 | 31.7 ± 2.1 |
| Reseeded | – | 51.3 ± 1.1 | 31.7 ± 1.6 | 32.2 ± 0.3 | 32.5 ±0.8 |
| Confluent | + | 76.2 ± 1.7 | 71.3 ± 0.9 | 45.9 ± 0.2 | 44.7 ± 0.4 |
| Confluent | – | 50.0 ± 2.6 | 32.4 ± 1.3 | 39.6 ± 0.2 | 36.3 ± 0.2 |

[a] Cultures in 100 mm Petri dishes were treated with 10 mL each of 10 μg/mL asbestos and 3 μg/mL $^3$H-BaP for 8 hr. Each experiment was done in triplicate and expressed as value ± SD.

Table 2. Recovery of organic and water-soluble $^3$H-BaP metabolites from culture medium of human fibroblasts.[a]

| Culture | Serum | Radioactivity in organic phase, % | | Radioactivity in aqueous phase, % | |
|---|---|---|---|---|---|
| | | Control | Chrysotile | Control | Chrysotile |
| Reseeded | + | 69.7 ± 0.8 | 64.6 ± 0.8 | 9.5 ± 0.2 | 9.0 ± 0.5 |
| Reseeded | – | 82.2 ± 0.7 | 78.1 ± 4.3 | 14.8 ± 1.0 | 20.9 ± 1.6 |
| Confluent | + | 44.9 ± 0.6 | 47.9 ± 2.6 | 11.4 ± 0.6 | 11.8 ± 0.7 |
| Confluent | – | 69.9 ± 1.9 | 70.1 ± 5.5 | 27.4 ± 1.5 | 33.7 ± 1.0 |

[a] Extraction was done with equal volumes of acetone to denature the proteins and then further extracted with additional equal volumes of ethyl acetate (1:1:1); in (value ± SD).

serum plus medium may be due to an association, either covalently or noncovalently, of the BaP or its metabolites with denatured proteins which settled at the interphase. In the presence of serum, there was no observable difference in the amount of water-soluble metabolites recovered between the control and the asbestos-treated cultures. When serum was withheld from the growth medium, a significantly higher percentage of radioactivity was recovered in the aqueous phase of the asbestos-treated medium, indicating that more BaP was metabolized in the treated culture than the control. The initial metabolic activation of BaP is carried out by the cytochrome P-450 enzyme system (*14*). A minute portion of the metabolites is eventually transformed to electrophiles which covalently bind to any available nucleophilic centers on cellular macromolecules, thereby forming adducts. Since cellular proteins and RNAs are constantly turned over, only DNA modifications (e.g., DNA adducts) are assumed to be involved in the manifestation of the long term effects of such damage. Those DNA adducts not removed prior to DNA replication are believed to lead to an infidelity of DNA replication which is subsequently passed on in the genetic material to the daughter cell (*11, 15*).

The BaP-RNA and the BaP-DNA binding of normal human skin fibroblasts in the presence or absence of chrysotile treatment are summarized in Table 3. In the presence of serum, the BaP-nucleic acid binding is not modified by asbestos treatment, while under serum-free cultures, there is a significant enhancement of BaP-nucleic acid binding in the presence of chrysotile for both the reseeded and confluent cultures. The observed enhancement in binding is interpreted to mean simply that more BaP is available intracellularly as a substrate for the cytochrome P-450 enzymes. The binding data substantiate the observation that under serum-free conditions there is an increased uptake of BaP in the presence of chrysotile. Previously, it was demonstrated that chrysotile can adsorb BaP easily in the absence of serum and the adsorbed BaP was rapidly eluted upon the addition of serum suggesting that under serum-free conditions, chrysotile acts as a vehicle for bringing BaP into cells (*7*). This does not, however,

Table 3. Chrysotile effects on $^3$H-BaP-nucleic acid binding in normal human fibroblasts.[a]

| Culture | Serum | $CPM_{260}$ treated / $CPM/OD_{260}$ control | |
|---|---|---|---|
| | | RNA | DNA |
| Reseeded | + | 1.03 ± 0.16 | 1.15 ± 0.10 |
| Reseeded | – | 1.29 ± 0.39 | 2.76 ± 0.33 |
| Confluent | + | 0.99 ± 0.03 | 1.00 ± 0.17 |
| Confluent | – | 1.72 ± 0.41 | 2.21 ± 0.48 |

[a] RNA was hydrolyzed with 0.3 *N* KOH at 37°C for 18 hr before DNA was extracted with 5% PCA at 80°C for 45 min; in value ± SD.

appear to be the case under serum-added conditions, which are more representative of *in vivo* conditions.

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